EXHIBIT 4

GNE.3230R1C39

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Eaton, et al.
Appl. No.	:	10/063,557
Filed	:	May 2, 2002
For	:	SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME
Examiner	:	David J. Blanchard
Group Art Unit	:	1642

DECLARATION OF J. CHRISTOPHER GRIMALDI, UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, J. Christopher Grimaldi, declare and say as follows:

1. I am a Senior Research Associate in the Molecular Biology Department of Genentech, Inc., South San Francisco, CA 94080.

2. I joined Genentech in January of 1999. From 1999 to 2003, I directed the Cloning Laboratory in the Molecular Biology Department. During this time I directed or performed numerous molecular biology techniques including qualitative Polymerase Chain Reaction (PCR) analyses. I am currently involved in, among other projects, the isolation of genes coding for membrane associated proteins which can be used as targets for antibody therapeutics against cancer. In connection with the above-identified patent application, I personally performed or directed the semi-quantitative PCR analyses in the assay entitled "Tumor Versus Normal Differential Tissue Expression Distribution" which is described in EXAMPLE 18 in the specification that were used to identify differences in gene expression between tumor tissue and their normal counterparts.

3. My scientific Curriculum Vitae, including my list of publications, is attached to and forms part of this Declaration (Exhibit A).

4. In differential gene expression studies, one looks for genes whose expression levels differ significantly under different conditions, for example, in normal versus diseased tissue.

Chromosomal aberrations, such as gene amplification, and chromosomal translocations are important markers of specific types of cancer and lead to the aberrant expression of specific genes and their encoded polypeptides, including over-expression and under-expression. For example, gene amplification is a process in which specific regions of a chromosome are duplicated, thus creating multiple copies of certain genes that normally exist as a single copy. Gene under-expression can occur when a gene is not transcribed into mRNA. In addition, chromosomal translocations occur when two different chromosomes break and are rejoined to each other chromosome resulting in a chimeric chromosome which displays a different expression pattern relative to the parent chromosomes. Amplification of certain genes such as Her2/Neu [Singleton *et al.*, Pathol. Annu., 27Pt1:165-190], or chromosomal translocations such as t(5;14), [Grimaldi *et al.*, Blood, 73(8):2081-2085(1989); Meeker *et al.*, Blood, 76(2):285-289(1990)] give cancer cells a growth or survival advantage relative to normal cells, and might also provide a mechanism of tumor cell resistance to chemotherapy or radiotherapy. When the chromosomal aberration results in the aberrant expression of a mRNA and the corresponding gene product (the polypeptide), as it does in the aforementioned cases, the gene product is a promising target for cancer therapy, for example, by the therapeutic antibody approach.

5. Comparison of gene expression levels in normal versus diseased tissue has important implications both diagnostically and therapeutically. For example, those who work in this field are well aware that in the vast majority of cases, when a gene is over-expressed, as evidenced by an increased production of mRNA, the gene product or polypeptide will also be over-expressed. It is unlikely that one identifies increased mRNA expression without associated increased protein expression. This same principle applies to gene under-expression. When a gene is under-expressed, the gene product is also likely to be under-expressed. Stated in another way, two cell samples which have differing mRNA concentrations for a specific gene are expected to have correspondingly different concentration of protein for that gene. Techniques used to detect mRNA, such as Northern Blotting, Differential Display, *in situ* hybridization, quantitative PCR, Taqman, and more recently Microarray technology all rely on the dogma that a change in mRNA will represent a similar change in protein. If this dogma did not hold true then these techniques would have little value and not be so widely used. The use of mRNA quantitation techniques have identified a seemingly endless number of genes which are differentially expressed in various tissues and these genes have subsequently been shown to have correspondingly similar changes in their protein levels. Thus, the detection of increased mRNA expression is expected to result in increased polypeptide expression, and the detection of decreased mRNA expression is expected to result in decreased polypeptide expression. The detection of increased or decreased polypeptide expression can be used for cancer diagnosis and treatment.

6. However, even in the rare case where the protein expression does not correlate with the mRNA expression, this still provides significant information useful for cancer diagnosis and treatment. For example, if over- or under-expression of a gene product does not correlate with over- or under-expression of mRNA in certain tumor types but does so in others, then identification of both gene expression and protein expression enables more accurate tumor classification and hence better determination of suitable therapy. In addition, absence of over- or

under-expression of the gene product in the presence of a particular over- or under-expression of mRNA is crucial information for the practicing clinician. For example, if a gene is over-expressed but the corresponding gene product is not significantly over-expressed, the clinician accordingly will decide not to treat a patient with agents that target that gene product.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By: _____ Date: 8/10/2004
J. Christopher Grimaldi

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J. Christopher Grimaldi

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San Francisco, CA 94122
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EDUCATION

University of California, Berkeley
Bachelor of Arts in Molecular Biology, 1984

EMPLOYMENT EXPERIENCE

SRA — Genentech Inc., South San Francisco; 1/99 to present

Previously, was responsible to direct and manage the Cloning Lab. Currently focused on isolating cancer specific genes for the Tumor Antigen (TAP), and Secreted Tumor Protein (STOP) projects for the Oncology Department as well as Immunologically relevant genes for the Immunology Department. Directed a lab of 6 scientists focused on a company-wide team effort to identify and isolate secreted proteins for potential therapeutic use (SPDI). For the SPDI project my duties were, among other things, the critically important coordination of the cloning of thousands of putative genes, by developing a smooth process of communication between the Bioinformatics, Cloning, Sequencing, and Legal teams. Collaborated with several groups to discover novel genes through the Curagen project, a unique differential display methodology. Interacted extensively with the Legal team providing essential data needed for filing patents on novel genes discovered through the SPDI, TAP and Curagen projects. My group has developed, implemented and patented high throughput cloning methodologies that have proven to be essential for the isolation of hundreds of novel genes for the SPDI, TAP and Curagen projects as well as dozens of other smaller projects.

Scientist — DNAX Research Institute, Palo Alto; 9/91 to 1/99

Involved in multiple projects aimed at understanding novel genes discovered through bioinformatics studies and functional assays. Developed and patented a method for the specific depletion of eosinophils in vivo using monoclonal antibodies. Developed and implemented essential technical methodologies and provided strategic direction in the areas of expression, cloning, protein purification, general molecular biology, and monoclonal antibody production. Trained and supervised numerous technical staff.

Facilities Manager — Corixa, Redwood City; 5/89 - 7/91.

Directed plant-related activities, which included expansion planning, maintenance, safety, purchasing, inventory control, shipping and receiving, and laboratory management. Designed and implemented the safety program. Also served as liaison to regulatory agencies at the local, state and federal level. Was in charge of property leases, leasehold improvements, etc. Negotiated vendor contracts and directed the purchasing department. Trained and supervised personnel to carry out the above-mentioned duties.

SRA — University of California, San Francisco
Cancer Research Institute; 2/87-4/89.

Was responsible for numerous cloning projects including: studies of somatic hypermutation, studies of AIDS-associated lymphomas, and cloning of t(5;14), t(11;14), and t(8;14) translocations. Focused on the activation of hemopoietic growth factors involved in the t(5;14) translocation in leukemia patients..

Research Technician — Berlex Biosciences, South San Francisco; 7/85-2/87.

Worked on a subunit porcine vaccine directed against Mycoplasma hyopneumoniae. Was responsible for generating genomic libraries, screening with degenerate oligonucleotides, and characterizing and expressing clones in E. coli. Also constructed a general purpose expression vector for use by other scientific teams.

PUBLICATIONS

1. Hilary P. Clark, et al. "The Secreted Protein Discovery Initiative (SPDI), a Large-scale Effort to Identify Novel Human Secreted and Transmembrane Proteins: a bioinformatics assessment." Genome Res. Vol 13(10), 2265-2270, 2003

2. Sean H. Adams, Clarissa Chui' Sarah L. Schilbach, Xing Xian Yu, Audrey D. Goddard, J. Christopher Grimaldi, James Lee, Patrick Dowd, David A. Lewin, & Steven Colman' "BFIT, a Unique Acyl-CoA Thioesterase Induced in Thermogenic Brown Adiopose Tissue: Cloning, organization of the humanb gene and assessment of a potential link to obesity" Biochemical Journal, Vol 360, 135-142, 2001

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4. Jeanne Kahn, Fuad Mehraban, Gladdys Ingle, Xiaohua Xin, Juliet E. Bryant, Gordon Vehar, Jill Schoenfeld, J. Christopher Grimaldi (incorrectly named as "Grimaldi, CJ"), Franklin Peale, Aparna Draksharapu, David A. Lewin, and Mary E. Gerritsen. "Gene Expression Profiling in an in Vitro Model of Angiogenesis." American Journal of Pathology Vol 156(6), 1887-1900, 2000.

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Division into Peripheral Monoclonal, Polyclonal and Central Nervous System Lymphoma." AIDS, Vol. 5, 669-674, 1991

18. Ann Grimaldi and Chris Grimaldi. "Small-Scale Lambda DNA Prep." Contribution to Current Protocols in Molecular Biology, Supplement 5, Winter 1989

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21 Timothy C. Meeker, J. Christopher Grimaldi, Robert O'Rourke, et al. "Lack of Detectable Somatic Hypermutation in the V Region of the Ig H Chain Gene of a Human Chronic B Lymphocytic Leukemia." The Journal of Immunology, Vol. 141, 3994-3998, 1988

MANUSCRIPTS IN PREPARATION

1. Sriram Balasubramanian, J. Christopher Grimaldi, J. Fernando Bazan, Gerard Zurawski and Maureen Howard. "Structural and functional characterization of CD38: Identification of active site residues"

PATENTS

1. "Methods for Eosinophil Depletion with Antibody to CCR3 Receptor" (US 6,207,155 B1).

2. "Amplification Based Cloning Method." (US 6,607,899)

3. Ashkenazi et al., "Secreted and Transmembrane Polypeptides and Nucleic Acids Encoding the Same." (this patent covers several hundred genes)

4. "IL-17 Homologous Polypeptides and Therapeutic Uses Thereof"

5. "Method of Diagnosing and Treating Cartilaginous Disorders."

MEMBERSHIPS AND ACTIVITIES

Editor	Frontiers in Bioscience
Member	DNAX Safety Committee 1991-1999 Biological Safety Affairs Forum (BSAF) 1990-1991 Environmental Law Foundation (ELF) 1990-1991

The t(5;14) Chromosomal Translocation in a Case of Acute Lymphocytic Leukemia Joins the Interleukin-3 Gene to the Immunoglobulin Heavy Chain Gene

By J. Christopher Grimaldi and Timothy C. Meeker

Chromosomal translocations have proven to be important markers of the genetic abnormalities central to the pathogenesis of cancer. By cloning chromosomal breakpoints one can identify activated proto-oncogenes. We have studied a case of B-lineage acute lymphocytic leukemia (ALL) that was associated with peripheral blood eosinophilia. The chromosomal translocation t(5;14) (q31;q32) from this sample was cloned and studied at the molecular level. This translocation joined the immunoglobulin heavy chain joining (Jh) region to the promotor region of the interleukin-3 (IL-3) gene in opposite transcriptional orientations. The data suggest that activation of the IL-3 gene by the enhancer of the immunoglobulin heavy chain gene may play a central role in the pathogenesis of this leukemia and the associated eosinophilia.

KARYOTYPIC STUDIES of leukemia and lymphoma have identified frequent nonrandom chromosomal translocations. Some of these translocations juxtapose the immunoglobulin heavy chain (IgH) gene with important protoncogenes, such as c-*myc* and *bcl*-2.[1,2] In this way, the IgH gene can activate proto-oncogenes, resulting in disordered gene expression and a step in the development of cancer. The investigation of additional nonrandom translocations into the IgH locus allows us to identify new genes promoting the generation of leukemia and lymphoma.

A distinct subtype of acute lymphocytic leukemia (ALL) has been characterized by B-lineage phenotype, associated eosinophilia in the peripheral blood, and a t(5;14)(q31;q32) chromosomal translocation.[3,4] This syndrome probably occurs in <1% of all patients with ALL. We hypothesized that the cloning of the translocation characteristic of this leukemia might allow the identification of an important gene on chromosome 5 that plays a role in the evolution of this disease. In this report we demonstrate that the interleukin-3 gene (IL-3) and the IgH gene are joined by this translocation.

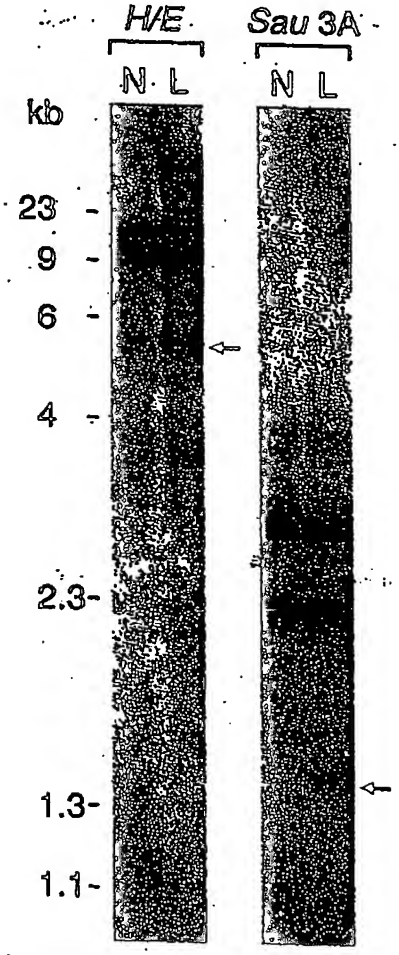

Fig 1. DNA blots of the leukemia sample. The restriction fragment pattern of normal human DNA (N) and the leukemia sample (L) were compared using a human Jh probe. Rearranged bands are indicated by arrows. Sample L exhibits a single rearranged band with both *Hind* III/*Eco*RI and *Sau*3A restriction digests. The rearranged bands are less intense than the other bands because the majority of cells in the sample represent normal bone marrow elements.

MATERIALS AND METHODS

Sample and DNA blots. A bone marrow aspirate from a representative patient with ALL (L1 morphology by French-American-British [FAB] criteria), peripheral eosinophilia (up to 20,000 per microliter with a normal value of <350 per microliter) and a t(5;14)(q31;q32) translocation was studied. Using published methods, genomic DNA was isolated and DNA blots were made.[5] Briefly, 10 μg of high molecular weight (mol wt) DNA were digested using an appropriate restriction enzyme and electrophoresed on a 0.8% agarose gel. The gel was stained with ethidium bromide, photographed, denatured, neutralized, and transferred to Hybond (Amersham, Arlington Heights, IL). After treatment of the filter with ultraviolet light, hybridization was performed. The filter was washed to a final stringency of 0.2% saturated sodium citrate (SSC) and 0.1% sodium lauryl sulfate (SDS) and exposed to film. The human Jh probe has been previously reported.[6]

Genomic library. The genomic library was made using pub-

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Dr Grimaldi's current address is Biostan Inc, 440 Chesapeake Dr, Seaport Centre, Redwood City, CA 94063.

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lished methods.[5] Approximately 100 μg of high mol wt genomic DNA were partially digested with the *Sau*3A restriction enzyme. Fragments from 9 to 23 kilobases (kb) in size were isolated on a sucrose gradient and ligated into phage EMBL3A (Strategene, San Diego). Recombinant phage were packaged, plated, and screened as previously reported.[5]

DNA sequencing. Fragments for sequencing were cloned into M13 vectors and sequenced by the chain termination method using Sequenase (United States Biochemical, Cleveland).[7] All sequence data were derived from both strands.

RESULTS

We studied a bone marrow sample from a patient with ALL and associated peripheral eosinophilia. Karyotypic analysis showed the characteristic t(5;14)(q31;q32) translocation. These features define a distinctive subtype of ALL.[3,4] The leukemic cells were analyzed for cell surface phenotype by immunofluorescence. They were positive for B1 (CD20), B4 (CD19), cALLA (CD10), HLA-DR, and terminal deoxynucleotidyl transferase (Tdt), but negative for surface immunoglobulin. This phenotypic profile describes an immature cell from the B-lymphocytic lineage.[8]

The leukemia DNA was analyzed by Southern blotting for rearrangements of the IgH gene. Using a human immunoglobulin Jh probe, a single rearranged band was detected by *Eco*RI, *Hind*III, *Sst*I, *Sau*3A, and *Eco*RI plus *Hind*III restriction digests, suggesting rearrangement of one allele (Fig 1). The immunoglobulin Jh region from the other allele was presumably either deleted or in the germline configuration.

We hypothesized that the t(5;14)(q31;q32) juxtaposed a growth-promoting gene on chromosome 5 with the immunoglobulin Jh region on chromosome 14. Therefore, a genomic library was made from the leukemic sample and screened with a Jh probe. Fifteen distinct positive clones were isolated and screened for the presence of the rearranged *Sau*3A fragment that was detected by DNA blotting. By this analysis, five clones appeared to represent the rearranged allele identified by DNA blots. One of these clones (clone no. 4) was chosen for further study and a detailed restriction map was generated. The *Eco*RI, *Hind*III/*Eco*RI, and *Sst*I fragments from clone no. 4 that hybridized to the human Jh probe were also identical in size to the rearranged fragments from the leukemia sample, confirming that clone no. 4 represented the rearranged leukemic allele.

Phage clone no. 4 contained 3.7 kb of unknown origin joined to the IgH gene in the region of Jh4 (Fig 2). The IgH gene from Jh4 to the Cmu region appeared to be in germline configuration. Previously, the gene encoding hematopoietic growth factor IL-3 had been mapped to chromosome 5q31 so it was suspected that clone no. 4 might contain part of this gene.[9-12] When the restriction map of human IL-3 and clone no. 4 were compared, they were identical for more than 3 kb (Fig 2).

We confirmed the juxtaposition of the IL-3 gene and the IgH gene by nucleic acid sequencing of the subcloned *Bst*EII/*Hpa*I fragment (Fig 2). The sequence of this fragment showed no disruption of the protein coding region or the messenger RNA of the IL-3 gene. The break in the IL-3 gene occurred in the promotor region, 452 base pairs (bp) upstream of the transcriptional start site (position 64, Fig

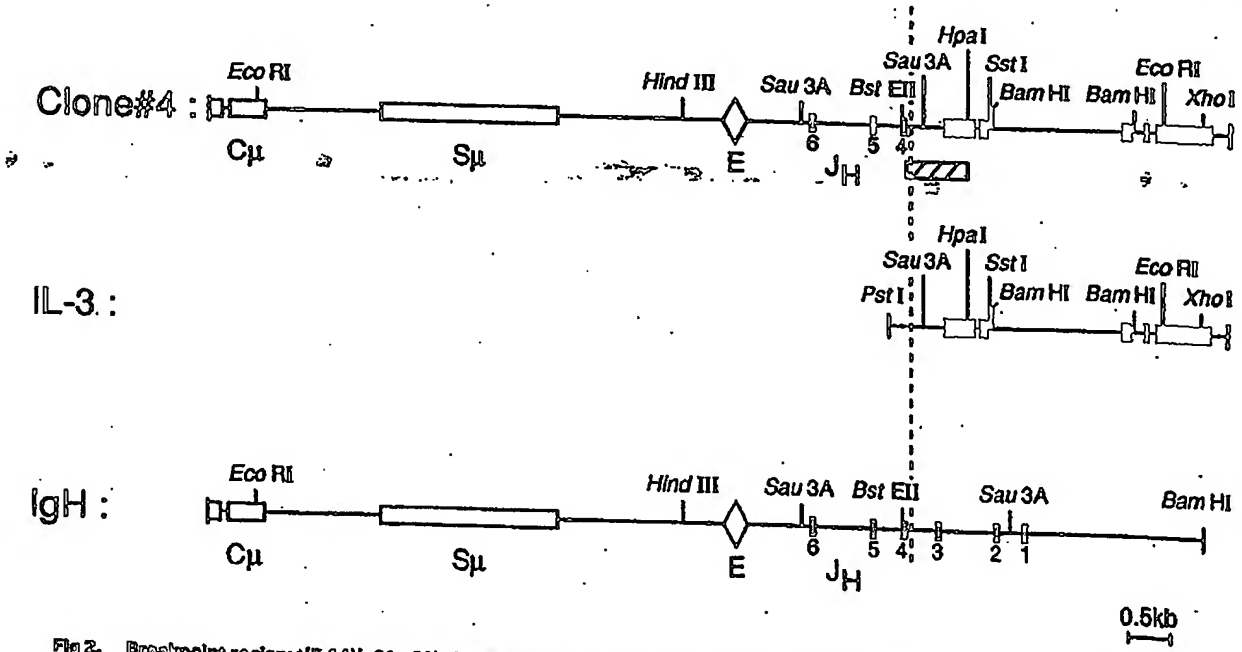

Fig 2. Breakpoint region: t(5;14)(q31;q32). Comparative mapping of phage clone no. 4, the germline IgJh region, and the germline IL-3 gene.[9,10] The map of clone no. 4 is identical to that of IgH until it diverges in the region of Jh4 (at the dashed line), after which it is identical to the map of IL-3. The two genes are positioned in a head-to-head orientation. The Ig μ chain constant region (Cμ), switch region (Sμ), enhancer (E), and Jh segments are indicated (open symbols). The five exons (dark boxes) and four introns of the IL-3 gene are shown. The hatched box indicates the sequenced region.

3A). The break in the IgH gene occurred 2 bp upstream of the Jh4 region. Between the two breaks, 25 bp of uncertain origin (putative N sequence) were inserted.[13,14] No sequences homologous to the immunoglobulin heptamer and nonamer could be identified in the IL-3 sequence (Fig 3B). Therefore, nucleic acid sequencing confirmed the juxtaposition of the IL-3 gene and the IgH gene. The sequence data clearly showed that the genes were positioned in opposite transcriptional orientations (head-to-head).

Available data also allowed us to determine the normal positions of the IL-3 gene and the GM-CSF gene in relation to the centromere of chromosome 5 (Fig 4). The IgH gene is known to be positioned with the variable regions toward the telomere on chromosome 14q.[2,15] It has also been shown that GM-CSF maps within 9 kb of IL-3 in the same transcriptional orientation.[16] Using this information and assuming a simple translocation event in our sample, we can conclude that the IL-3 gene is normally more centromeric, and the GM-CSF gene more telomeric on chromosome 5q (Fig 4). Furthermore, both are transcribed with their 5′ ends toward the centromere.

DISCUSSION

In this report we have cloned a unique chromosomal translocation that appears to be a consistent feature of a rare, yet distinct, clinical form of acute leukemia. This translocation joined the promotor of the IL-3 gene to the IgH gene. Except for the altered promotor, the IL-3 gene appeared

A

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5'GGTGACCAGGGTTCCCTGGCCCCAGTAGTCAAAGTAGTAGAGGTAATTCATCATAGCTGCGGATTAGCAGCGTGACCGGC
3'CCACTGGTCCCAAGGGACCGGGGTCATCAGTTTCATCATCTCCATTAAGTAGTATCGACGCCTAATCGTCGCACTGGCCG   80

5'TACCAGACAAACTCTCATCTGTTCCAGTGGCCTCCTGGCCACCCACCAGGACCAAGCAGGGCGGGCAGCAGAGGGCCAGG
3'ATGGTCTGTTTGAGAGTAGACAAGGTCACCGGAGGACCGGTGGGTGGTCCTGGTTCGTCCCGCCCGTCGTCTCCCGGTCC  160

5'GTAGTCCAGGTGATGGCAGATGAGATCCCACTGGGCAGGAGGCCTCAGTGAGCTGAGTCAGGCTTCCCCTTCCTGCCACA
3'CATCAGGTCCACTACCGTCTACTCTAGGGTGACCCGTCCTCCGGAGTCACTCGACTCAGTCCGAAGGGGAAGGACGGTGT  240

5'GGGGTCCTCTCACCTGCTGCCATGCTTCCCATCTCTCATCCTCCTTGACAAGATGAAGTGATACCGTTTAAGTAATCTTT
3'CCCCAGGAGAGTGGACGACGGTACGAAGGGTAGAGAGTAGGAGGAACTGTTCTACTTCACTATGGCAAATTCATTAGAAA  320

5'TTTCTTGTTTCACTGATCTTGAGTACTAGAAAGTCATGGATGAATAATTACGTCTGTGGTTTCTATGGAGGTTCCATGT
3'AAAGAACAAAGTGACTAGAACTCATGATCTTTCAGTACCTACTTATTAATGCAGACACCAAAAGATACCTCCAAGGTACA  400

5'CAGATAAAGATCCTTCCGACGCCTGCCCCACACCACCACCTCCCCCCGCCTTGCCCGGGGTTGTGGGCACCTTGCTGCTG
3'GTCTATTTCTAGGAAGGCTGCGGACGGGGTGTGGTGGTGGAGGGGGGCGGAACGGGCCCCAACACCCGTGGAACGACGAC  480

5'CACATATAAGGCGGGAGGTTGTTGCCAACTCTTCAGAGCCCCACGAAGGACCAGAACAAGACAGAGTGCCTCCTGCCGAT
3'GTGTATATTCCGCCCTCCAACAACGGTTGAGAAGTCTCGGGGTGCTTCCTGGTCTTGTTCTGTCTCACGGAGGACGGCTA  560

5'CCAAACATGAGCCGCCTGCCCGTCCTGCTCCTGCTCCAACTCCTGGTCCGCCCCGGACTCCAAGCTCCCATGACCCAGAC
3'GGTTTGTACTCGGCGGACGGGCAGGACGAGGACGAGGTTGAGGACCAGGCGGGGCCTGAGGTTCGAGGGTACTGGGTCTG  640

5'AACGTCCTTGAAGACAAGCTGGGTTAAC 3'
3'TTGCAGGAACTTCTGTTCGACCCAATTG 5'  668
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B

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IgJh4   5'TGGCCCCAGTAGTCAAAGTAGTCACATTGTGGGAGGCCCCATTAAGGGGTGCACAAAAACCTGACTCTC
        3'ACCGGGGTCATCAGTTTCATCAGTGTAACACCCTCCGGGGTAATTCCCCACGTGTTTTTGGACTGAGAG
           ++++++++++++++++++++++

Cl.04   5'TGGCCCCAGTAGTCAAAGTAGTAGAGGTAATTCATCATAGCTGCGGATTAGCAGCGTGACCGGCTACCA
        3'ACCGGGGTCATCAGTTTCATCATCTCCATTAAGTAGTATCGACGCCTAATCGTCGCACTGGCCGATGGT
                                                  ++++++++++++++++++++++

Il-3    5'GGCACCAAGAGATGTGCTTCTCAGAGCCTGAGGCTGAACGTGGATGTTTAGCAGCGTGACCGGCTACCA
        3'CCGTGGTTCTCTACACGAAGAGTCTCGGACTCCGACTTGCACCTACAAATCGTCGCACTGGCCGATGGT
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Fig 3. Sequence of t(5;14)(q31;q32) breakpoint region. (A) Nucleotide sequence of the *BstEII*/*Hpa*I fragment indicated on Fig 2. Nucleotides 1 to 39 represent the Jh4 coding region underlined on the coding strand.[9] Nucleotides 39 to 63 are a putative N region. The sequence from position 64 to 668 is that of the germline IL-3 gene.[20] The IL-3 TATA box (486), transcription start (515), and initiation methionine (587) are underlined. Two proposed regulatory sequences in the promotor are marked by asterisks (positions 182 and 389). (B) Comparative sequence of the t(5;14)(q31;q32) breakpoint region. The IgJh4 region is shown with its coding region, heptamer, and nonamer underlined. Clone no. 4 is shown with putative N region sequences underlined. The IL-3 sequence is also shown. A plus sign (+) denotes the identical nucleotide between sequences. No heptamer or nonamer is identified in the IL-3 sequence.

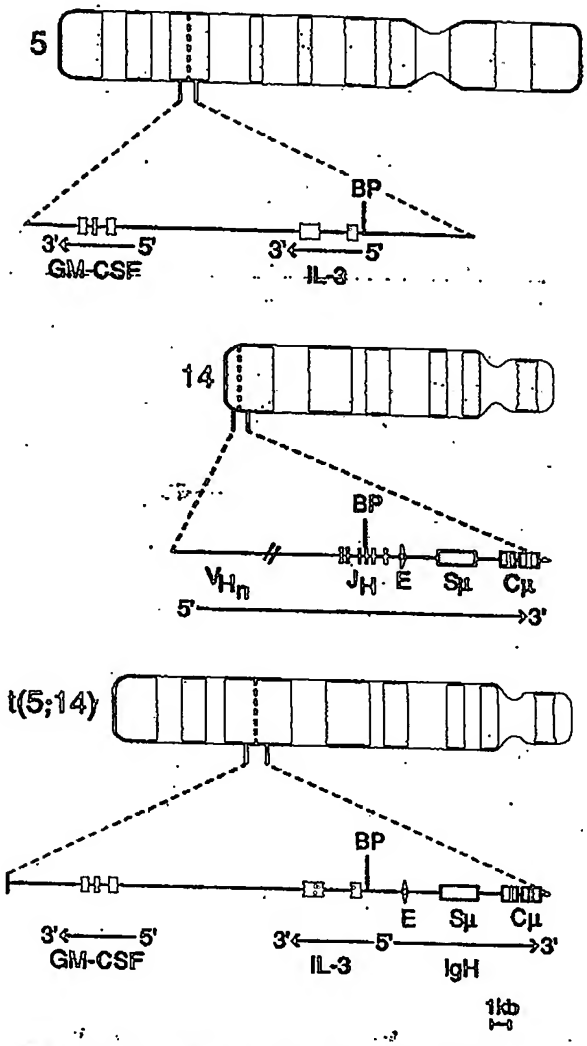

Fig 4. Diagram of the translocation. The normal chromosome 5q31 is shown with the GM-CSF gene telomeric to the IL-3 gene in the transcriptional orientation shown. On normal chromosome 14q32 the Vh regions are telomeric. The t(5;14)(q31;q32) translocation results in the head-to-head orientation of these genes. Symbols are defined in Fig 2. BP, breakpoint position.

intact as no deletions, insertions, or point mutations were detected by restriction mapping of the entire gene and sequencing of part of the gene. The IgH gene has been truncated at the Jh4 region, which places the immunoglobulin enhancer within 2.5 kb of the IL-3 gene.[17,18] This leads to the hypothesis that the enhancer is increasing transcription of a structurally normal IL-3 gene. The same mechanism is important for activation of the c-*myc* gene in some cases of Burkitt's lymphoma.[19] An alternate hypothesis is that the elimination of an upstream IL-3 promotor element is crucial to the activation of the IL-3 gene.

The proposed activation of the IL-3 gene suggests that an autocrine loop is important for the pathogenesis of this leukemia.[20] Over-expression of the IL-3 gene coupled with the presence of the IL-3 receptor in these cells could account for a strong stimulus for proliferation. In this regard, there are data indicating that immature B-lineage lymphocytes and B-lineage leukemias may express the IL-3 receptor.[21,22]

An additional feature of this type of leukemia is the dramatic eosinophilia, consisting of mature forms. It has been hypothesized that the eosinophils do not arise from the malignant clone, but are stimulated by the tumor.[20,20] Because of the known effect of IL-3 on eosinophil differentiation, secretion of high levels of IL-3 by leukemic cells might have a role in the eosinophilia in this type of leukemia.[23]

The data suggest that the recombination mechanism that is active in the IgH gene during normal differentiation has a role in this translocation.[13,14] This is supported by the breakpoint location at the 5' end of Jh4 and the presence of putative N-region sequences. On the other hand, no recombination signal sequence (heptamer and nonamer) was found in this region on chromosome 5, suggesting that additional factors also played a role. Further studies will elucidate the mechanism of this and other translocations.

In the leukemia we studied, it is possible that the immunoglobulin enhancer also activates the GM-CSF gene, since this gene is probably positioned only 14 kb away (Fig 4). This is known to be within the range of enhancer activation.[25] The interleukin-5 (IL-5) gene maps to chromosome 5q31.[26] Deregulation of the IL-5 gene by this translocation would act synergistically with IL-3 in the stimulation of eosinophil proliferation and differentiation.[27] These and other questions will be answered by the study of more patient samples. We plan to determine whether the t(5;14)(q31;q32) translocation is capable of activating multiple lymphokines simultaneously and whether they cooperate in the generation of this leukemia.

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RAPID COMMUNICATION

Activation of the Interleukin-3 Gene by Chromosome Translocation in Acute Lymphocytic Leukemia With Eosinophilia

By Timothy C. Meeker, Dan Hardy, Cheryl Willman, Thomas Hogan, and John Abrams

The t(5;14)(q31;q32) translocation from B-lineage acute lymphocytic leukemia with eosinophilia has been cloned from two leukemia samples. In both cases, this translocation joined the IgH gene and the interleukin-3 (IL-3) gene. In one patient, serum IL-3 mRNA was produced by the leukemic cells. In the second patient, serum IL-3 levels were measured and shown to correlate with disease activity. There was no evidence of excess granulocyte/macrophage colony stimulating factor (GM-CSF) or IL-5 expression. Our data support the formulation that this subtype of leukemia may arise in part because of a chromosome translocation that activates the IL-3 gene, resulting in autocrine and paracrine growth effects.

A NUMBER OF chromosome translocations have been associated with human leukemia and lymphoma. In many cases the study of these translocations has led to the discovery or characterization of proto-oncogenes, such as *bcl-2*, *c-abl*, and *c-myc*, that are located adjacent to the translocation.[1,2] It is now widely understood that cancer-associated translocations disrupt nearby proto-oncogenes.

A distinct subtype of acute leukemia is characterized by the triad of B-lineage immunophenotype, eosinophilia, and the t(5;14)(q31;q32) translocation.[3,4] Leukemic cells from such patients have been positive for terminal deoxynucleotidyl transferase (Tdt), common acute lymphoblastic leukemia antigen (CALLA), and CD19, but negative for surface or cytoplasmic immunoglobulin. In previous work, we cloned the t(5;14) breakpoint from one leukemic sample (Case 1) and determined that the IgH and interleukin-3 (IL-3) genes were joined by this abnormality.[5] In this report, we extend those findings by showing that the t(5;14)(q31;q32) translocation from a second leukemia sample (Case 2) has a similar structure, and we report our study of growth factor expression in these patients.

MATERIALS AND METHODS

Samples and Southern blots. Case 1 has been described.[5,6] Clinical features of Case 2 have been described in detail.[3] DNA isolation and Southern blotting was done using previously described methods.[5] Filters were hybridized with an immunoglobulin Jh probe, a 280 bp *Bam*HI/*Eco*RI genomic IL-3 fragment, and an IL-3 cDNA probe.[7,8]

Northern blots. RNA isolation and Northern blotting have been described.[9] Briefly, Northern blots were done by separating 9μg total RNA on 1% agarose-formaldehyde gels. Equal RNA loading in each lane was confirmed by ethidium bromide staining. Blots were hybridized with an IL-3 cDNA probe extending to the *Xho* I site in exon 5, a 720 bp *Sst* I/*Kpn* I probe derived from intron 2 of the IL-3 gene, a 600 bp *Nhe* I/*Hpa* II IL-5 cDNA probe, and a 500 bp *Pst* I/*Nco* I granulocyte-macrophage colony stimulating factor (GM-CSF) cDNA probe.[10-12]

Polymerase chain reaction. Primers were designed with *Bam*HI sites for cloning. One primer hybridized to the Jh sequences from the IgH gene (Primer 144:5'-TAGGATCCGACGGTGACCAGGGT), and the other hybridized to the region of the TATA box in the IL-3 gene (Primer 161: 5'-AACAGGATCCCGCCTTATATGTGCAG). Polymerase chain reaction (PCR) (95°C for 1 minute, 61°C for 30 seconds, and 72°C for 3 minutes) was done using 500 ng genomic DNA and 50 pmol of each primer in 100 μL containing 67 mmol/L Tris-HCl pH 8.8, 6.7 mmol/L $MgCl_2$, 10% dimethyl sulfoxide (DMSO), 170 μg/mL bovine serum albumin (BSA) (fraction V), 16.6 mmol/L ammonium sulfate, 1.5 mmol/L each dNTP and Taq polymerase (Perkin-Elmer, Norwalk, CT).[13]

Sequencing. Sequencing was done by chain termination in M13 vectors.[14] As part of this study, we sequenced a subclone of a normal IL-3 promotor, covering 598 base pairs from a *Sma* I site at position −1240 (with respect to the proposed site of transcription initiation) to an *Nhe* I site at position −642. The plasmid containing this region was a gift from Naoko Arai of the DNAX Research Institute.

Expression in Cos7 cells. A genomic IL-3 fragment from Case 1 was cloned into the pXM expression vector.[10] Briefly, the *Hin*dIII/*Sal* I fragment containing the IL-3 gene was subcloned from the previously described phage clone 4 into pUC18.[5] The 2.6 kb fragment extending from the *Sma* I site 61 bp upstream of the IL-3 transcription start to the *Sma* I site in the polylinker was cloned into the blunted *Xho* I site of pXM. The negative control construct was the pXM vector without insert. Plasmids were introduced into Cos7 cells by electroporation, and supernatant was collected after 48 hours in culture.

TF1 bioassay. TF-1 cells were passaged in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol L-glutamine, and 1 ng/mL human GM-CSF.[15] Samples and antibodies were diluted in this same medium lacking GM-CSF but containing penicillin and streptomycin. A 25 μL volume of serial dilutions of patient serum was added to wells in a flat bottom 96-well microtiter plate. Rat anti-cytokine monoclonal antibody in a volume of 25 μL was added to appropriate wells and preincubated for 1 hour at 37°C. Fifty microliters of twice washed TF-1 cells were added to each well, giving a final cell concentration of 1×10^5 cells per well (final volume, 100 μL). The plate was incubated for 48 hours. The remaining cell viability was determined metabolically by the colori-

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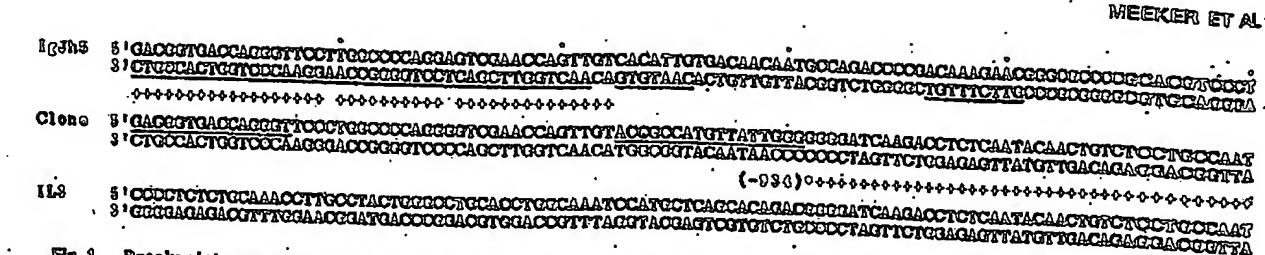

Fig 1. Breakpoint sequences for Case 2. The germline IgJh5 region sequence (protein coding region and recombination signal is in the middle, and the germline IL-3 sequence, which we derived from a normal IL-3 clone, is on the bottom. + indicates that each gene occurred at position −934 (°).

metric method of Mosmann using a VMax microtiter plate reader (Molecular Devices, Menlo Park, CA) set at 570 and 650 nm.[15]

Cytokine immunoassays. These assays used rat monoclonal anti-cytokine antibodies (10 μg/mL) to coat the wells of a PVC microtiter plate. The capture antibodies used were BVD3-5G8, JES1-39D10, and BVD2-23B6, for the IL-3, IL-5, and GM-CSF assays, respectively. Patient sera were then added (undiluted and diluted 1:2 for IL-3, undiluted for IL-5, and undiluted and diluted 1:5 for GM-CSF). The detecting immunoreagents used were either mouse antiserum to IL-3 or nitroiodophenyl (NIP)-derivatized rat monoclonal antibodies JES1-5A2 and BVD2-21C11, specific for IL-5 and GM-CSF, respectively. Bound antibody was subsequently detected with immunoperoxidase conjugates: horseradish peroxidase (HRP)-labeled goat anti-mouse Ig for IL-3, or HRP-labeled rat (J4 MoAb) anti-NIP for IL-5 and GM-CSF. The chromogenic substrate was 3-3'azino-bis-benzthiazoline sulfonate (ABTS; Sigma, St Louis, MO). Unknown values were interpolated from standard curves prepared from dilutions of the recombinant factors using Softmax software available with the VMAX microplate reader (Molecular Devices).

RESULTS

Leukemic DNA from Case 2 was studied by Southern blotting. When digested with the *Hin*dIII restriction enzyme and hybridized with a human immunoglobulin heavy chain joining region (Jh) probe, a rearranged fragment at approximately 14 kb was detected (data not shown). When reprobed with either of two different IL-3 probes, a rearranged 14 kb fragment, comigrating with the rearranged Jh fragment, was identified. When leukemic DNA was digested with *Hin*dIII plus *Eco*RI, a rearranged Jh fragment was detected at 6 kb. The IL-3 probes also identified a comigrating fragment of this size. These experiments indicated that the leukemic sample studied was clonal and that a single fragment contained both Jh and IL-3 sequences, suggesting a translocation had occurred.

To characterize better the joining of the IL-3 gene and the immunoglobulin heavy chain (IgH) gene, the polymerase chain reaction (PCR) was used to clone the translocation.[16] A Jh primer and an IL-3 primer were designed to produce an amplified product in the event of a head-to-head translocation. While control DNA gave no PCR product, Case 2 DNA yielded a PCR-derived fragment of approximately 980 bp, which was cloned and sequenced.

The DNA sequence of the translocation clone from Case 2 confirmed the joining of the Jh region with the promotor of the IL-3 gene in a head-to-head configuration (Fig 1). Sequence analysis indicated that the breakpoint on chromosome 14 was just upstream of the Jh5 coding region. The breakpoint on chromosome 5 occurred 934 bp upstream of the putative site of transcription initiation of the IL-3 gene. We also determined that a putative N sequence of 17 bp was inserted between the chromosome 5 and chromosome 14 sequences during the translocation event.[17,18] Figure 2 shows

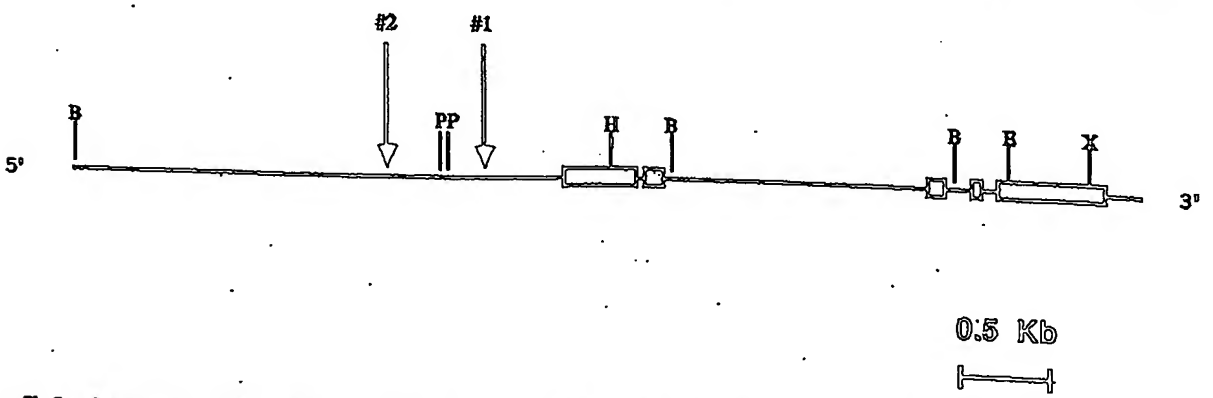

Fig 2. Relationship of chromosome 5 breakpoints to the IL-3 gene. This figure shows the two cloned breakpoints (arrows) in relation to the normal IL-3 gene.[9,10] One breakpoint occurred at position −462 and the other at −934 (arrows). In both circumstances, the translocations resulted in a head-to-head joining of the IgH gene and the IL-3 gene, leaving the mRNA and protein coding regions of the IL-3 gene intact. Boxes denote the five IL-3 exons; restriction enzymes are (B) *Bam*H, (P) *Pst* I, (H) *Hpa* I, (E) *Eco*RI, and (X) *Xho* I.

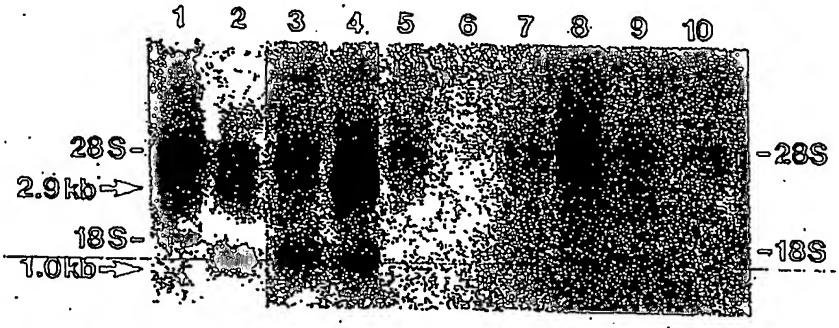

Fig 3. Documentation of IL-3 mRNA over-expression. A Northern blot was prepared and hybridized with a probe for IL-3. Lane 1 contained RNA from unstimulated peripheral blood lymphocytes (PBL) as a negative control. Lane 2 contained RNA from PBL stimulated for 4 hours with concanavalin A (ConA), and lane 3 contained RNA from PBL stimulated with ConA for 48 hours. As in the positive control lanes (2 and 3), a 1 kb band was identified in the leukemic sample from Case 1 (lane 4, lower arrow), suggesting aberrant expression of the IL-3 gene. In addition, the leukemic sample showed over-expression of an unspliced 2.9 kb IL-3 transcript (lane 4, upper arrow). We documented that this represented an unspliced precursor of the mature 1 kb transcript by showing that this band hybridized to a probe from intron 2 of the IL-3 gene. A similar 2.9 kb band was detected in lane 2, suggesting that an IL-3 mRNA of this size is sometimes detectable in normal mitogen-stimulated cells. Lane 5 through 10 represent RNA from six samples of B-lineage acute lymphocytic leukemia without the t(5;14) translocation, indicating that only the sample with the translocation exhibited IL-3 over-expression. Case 2 could not be analyzed by Northern blot because too few cells were available for study.

the locations of the two cloned breakpoints in relation to the IL-3 gene. The two chromosome 5 breakpoints were separated by less than 500 bp.

The genomic structure in Cases 1 and 2 suggested that a normal IL-3 gene product was over-expressed as a result of the altered promotor structure. This would predict that the IL-3 gene on the translocated chromosome was capable of making IL-3 protein. This prediction was tested by expressing a genomic fragment from the translocated allele of Case 1 containing all five IL-3 exons under the control of the SV40 promotor/enhancer in the Cos7 cell line. Cell supernatants were studied in a proliferation assay using the factor dependent erythroleukemic cell line, TF-1. The supernatants derived from transfections using the vector plus insert supported TF-1 proliferation, while supernatants from transfections using the vector alone were negative in this assay (data not shown). Furthermore, the biologic activity could be blocked by an antibody to human IL-3 (BVD3-6G8). This result showed that the translocated allele retained the ability to make IL-3 mRNA and protein.

The level of expression of IL-3 mRNA in leukemic cells from Case 1 was assessed. Northern blotting showed that the mature IL-3 mRNA (approximately 1 kb) and a 2.9 kb unspliced IL-3 mRNA were excessively produced by the leukemia (Fig 3). The 2.9 kb form of the mRNA is also present at low levels in normal peripheral blood T lymphocytes after mitogen activation (Fig 3). Several B-lineage acute leukemia samples without the t(5;14) translocation had undetectable levels of IL-3 mRNA in these experiments. In addition, although genes for GM-CSF and IL-5 map close to the IL-3 gene and might have been deregulated by the translocation, no IL-5 or GM-CSF mRNA could be detected in the leukemic sample (data not shown).[19,20]

Three serum samples from Case 2 were assayed by immunoassay for levels of IL-3, GM-CSF, and IL-5 (Table 1). Serum IL-3 could be detected and correlated with the clinical course. When the patient's leukemic cell burden was highest, the IL-3 level was highest. No serum GM-CSF or IL-5 could be detected.

Since the IL-3 immunoassay measured only immunoreactive factor, we confirmed that biologically active IL-3 was present by using the TF-1 bioassay. This bioassay can be rendered monospecific using appropriate neutralizing monoclonal antibodies specific for IL-3, IL-5, or GM-CSF. We observed that sera from 1-16-84 and 3-14-84 contained TF-1 stimulating activity that could be blocked with anti–IL-3 MoAb (BVD3-6G8), but not with MoAbs to IL-5 (JES1-39D10) or GM-CSF (BVD2-23B6) (Fig 4; GM-CSF data not shown). The amount of neutralizable bioactivity in these two samples correlated very well with the difference in IL-3 levels obtained by immunoassay for these samples. Furthermore, the failure to block TF-1 proliferating activity with either anti–IL-5 or anti–GM-CSF was consistent with the inability to measure these factors by immunoassay and

Table 1. Peripheral Blood Counts and Growth Factor Levels at Different Times in Case 2

	Sample Date		
	11/15/83	1/16/84	3/14/84
Peripheral blood counts (cells/μL)			
WBC	81,800	116,500	12,300
Lymphoblasts	0	33,785	0
Eosinophils	46,826	73,080	615
Serum growth factor levels (pg/mL)			
IL-3	<444	7,995	1,051
GM-CSF	<15	<15	<15
IL-5	<50	<50	<50

Peripheral blood counts from Case 2 at three different time points with the corresponding growth factor levels quantified by immunoassay. The patient received chemotherapy between 1/16/84 and 3/14/84 to lower his leukemic burden.[3] No serum samples were available for a similar analysis of Case 1.

Abbreviation: WBC, white blood cells.

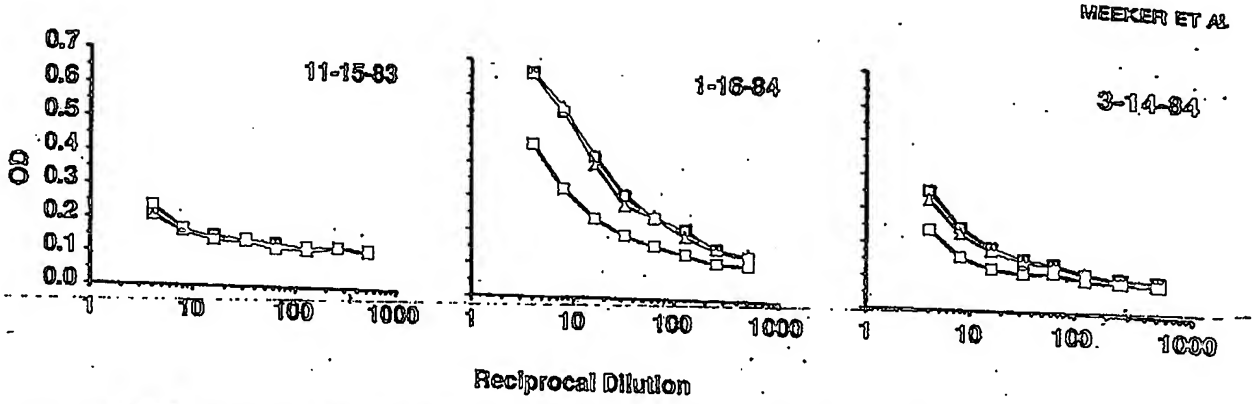

Fig 4. Bioassay of serum IL-3. Leukemic patient sera were tested for bioactive IL-3 and IL-5 in the TF-1 proliferation assay. The reciprocal of the dilution is indicated on the horizontal axis and the optical density indicating the amount of proliferation is indicated on the vertical axis. Serum from all three time points was assayed simultaneously. The assay was rendered monospecific by using a 1 μg/mL final concentration of monoclonal rat anti–IL-3, BVD3-6G3 (□), or anti–IL-5, JES1-39D10 (△); □ indicates no MoAb. On 11/10/83 and 3/14/84, inhibition of proliferation was evident in the presence of anti–IL-3 antibody, documenting serum levels of IL-3 on these days. Serum IL-5 was not detected in this assay, as anti–IL-5 did not alter TF-1 proliferation.

indicated that these other myeloid growth factors were not detectably circulating in the serum of this patient.

DISCUSSION

In this report, we have extended our analysis of acute lymphocytic leukemia and eosinophilia associated with the t(5;14) translocation. In both cases we have studied, we have documented the joining of the IL-3 gene from chromosome 5 to the IgH gene from chromosome 14. The breakpoints on chromosome 5 are within 500 bp of each other, suggesting that additional breakpoints will be clustered in a small region of the IL-3 promotor. The PCR assay we have developed will be useful in the screening of additional clinical samples for this abnormality.

The finding of a disrupted IL-3 promotor associated with an otherwise normal IL-3 gene implied that this translocation might lead to the over-expression of a normal IL-3 gene product. In this work, we have documented that this is true. In addition, neither GM-CSF nor IL-5 are over-expressed by the leukemic cells. Furthermore, in one patient, serum IL-3 could be measured and correlated with disease activity. To our knowledge, this is the first measurement of human IL-3 in serum and its association with a disease process. The measurement of serum IL-3 in this and other clinical settings may now be indicated.

The finding of the IL-3 gene adjacent to a cancer-associated translocation breakpoint suggests that its activation is important for oncogenesis. It is our thesis that an autocrine loop for IL-3 is important for the evolution of this leukemia.[21] The excessive IL-3 production that we have documented would be one feature of such an autocrine loop. The final proof of our thesis must await additional data. In particular, from the study of additional clinical samples, it will be necessary to document that the IL-3 receptor is present on the leukemic cells and that anti–IL-3 antibody decreases proliferation of the leukemia in vitro.

An important aspect of this work is the suggestion of a therapeutic approach for this disease. If an autocrine loop for IL-3 can be documented in this disease, attempts to lower circulating IL-3 levels or block the interaction of IL-3 with its receptor may prove useful. Because it is also possible that the eosinophilia in these patients is mediated by the paracrine effects of leukemia-derived IL-3, similar interventions may improve this aspect of the disease. Antibodies or engineered ligands to accomplish these goals may soon be available.

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Clinical and Pathologic Significance of the c-*erb*B-2 (*HER*-2/*neu*) Oncogene

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The c-*erb*B-2 oncogene was first shown to have clinical significance in 1987 by Slamon et al,[79] who reported that c-*erb*B-2 DNA amplification in breast carcinomas correlated with decreased survival in patients with metastasis to axillary lymph nodes. Subsequent studies, however, of c-*erb*B-2 activation in breast carcinoma reached conflicting conclusions about its clinical significance. This oncogene also has been reported to have clinical and pathologic implications in other neoplasms. Our review summarizes these various studies and examines the clinical relevance of c-*erb*B-2 activation, which has not been emphasized in recent reviews.[37,53,55] The molecular biology of the c-*erb*B-2 oncogene has been extensively reviewed[37,53,55] and will be discussed only briefly here.

BACKGROUND

The c-*erb*B-2 oncogene was discovered in the 1980s by three lines of investigation. The *neu* oncogene was detected as a mutated transforming gene in neuroblastomas induced by ethylnitrosurea treatment of fetal rats.[8,73,74,75] The c-*erb*B-2 was a human gene discovered by its homology to the retroviral gene v-*erb*B.[33,49,76] *HER*-2 was isolated by screening a human genomic DNA library for homology with v-*erb*B.[24] When the DNA sequences were determined subsequently, c-*erb*B-2, *HER*-2, and *neu* were found to represent the same gene. Recently, the c-*erb*B-2 oncogene also has been referred to as *NGL*.

The c-*erb*B-2 DNA is located on human chromosome 17q21[24,33,68] and codes for c-*erb*B-2 mRNA (4.6 kb), which translates c-*erb*B-2 protein (p185). This

protein is a normal component of cytoplasmic membranes. The c-*erb*B-2 oncogene is homologous with, but not identical to, c-*erb*B-1, which is located on chromosome 7 and codes for the epidermal growth factor receptor.[1,103] The c-*erb*B-2 protein is a receptor on cell membranes and has intracellular tyrosine kinase activity and an extracellular binding domain.[2,103] Electron microscopy with a polyclonal antibody detects c-*erb*B-2 immunoreactivity on cytoplasmic membranes of neoplasms, especially on microvilli and the non-villous outer cell membrane.[60] In normal cells, immunohistochemical reactivity for c-*erb*B-2 is frequently present at the basolateral membrane or the cytoplasmic membrane's brush border.[20,60]

There is experimental evidence that c-*erb*B-2 protein may be involved in the pathogenesis of breast neoplasia. Overproduction of otherwise normal c-*erb*B-2 protein can transform a cell line into a malignant phenotype.[27] Also, when the *neu* oncogene containing an activating point mutation is placed in transgenic mice with a strong promoter for increased expression, the mice develop multiple independent mammary adenocarcinomas.[12,63] In other experiments, monoclonal antibodies against the *neu* protein inhibit the growth (in nude mice) of a *neu*-transformed cell line,[30–32] and immunization of mice with *neu* protein protects them from subsequent tumor challenge with the *neu*-transformed cell line.[14] Some authors have speculated that the use of antagonists for the unknown ligand could be useful in future chemotherapy.[35] Further review of this experimental evidence is beyond the scope of this article.

The c-*erb*B-2 activation most likely occurs at an early stage of neoplastic development. This hypothesis is supported by the presence of c-*erb*B-2 activation in both in situ and invasive breast carcinomas. In addition, studies of metastatic breast carcinomas usually demonstrate uniform c-*erb*B-2 activation at multiple sites in the same patient,[11,18,38,41,52] although c-*erb*B-2 activation has rarely been detected in metastatic lesions but not in the primary tumor.[57,63,107] Even more rarely, c-*erb*B-2 DNA amplification has been detected in a primary breast carcinoma but not in its lymph node metastasis.[5] In patients who have bilateral breast neoplasms, both lesions have similar patterns of c-*erb*B-2 activation, but only a few such cases have been studied.[11]

MECHANISMS OF c-*erb*B-2 ACTIVATION

The most common mechanism of c-*erb*B-2 activation is genomic DNA amplification, which almost always results in overproduction of c-*erb*B-2 mRNA and protein.[17,54,63,81] The c-*erb*B-2 amplification may stabilize the overproduction of mRNA or protein through unknown mechanisms. Human breast carcinomas with c-*erb*B-2 amplification contain 2 to 40 times more c-*erb*B-2 DNA[4,9] and 4 to 128 times more c-*erb*B-2 mRNA[34,69] than found in normal tissue. Most human breast carcinomas with c-*erb*B-2 amplification have 2 to 15 times more c-*erb*B-2 DNA. Tumors with greater amplification tend to have greater overproduction.[17,53,63] The non-mammary neoplasms that have been studied tend to have

similar levels of c-*erb*B-2 amplification or overproduction relative to the corresponding normal tissue.

The second most common mechanism of c-*erb*B-2 activation is overproduction of c-*erb*B-2 mRNA and protein without amplification of c-*erb*B-2 DNA.[51] The quantities of mRNA and protein usually are less than those in amplified cases and may approach the small quantities present in normal breast or other tissues.[17,52,53] The c-*erb*B-2 protein overproduction without mRNA overproduction or DNA amplification has been described in a few human breast carcinoma cell lines.[47]

Other rare mechanisms of c-*erb*B-2 activation have been reported. Translocations involving the c-*erb*B-2 gene have been described in a few mammary and gastric carcinomas, although some reported cases may represent restriction fragment length polymorphisms or incomplete restriction enzyme digestions that mimic translocations.[51,63,74,6,78,100] A single point mutation in the transmembrane portion of *neu* has been described in rat neuroblastomas induced by ethylnitrosurea.[9,65] The mutated *neu* protein has increased tyrosine kinase activity and aggregates at the cell membrane.[12,13,65] Although there has been speculation that some of the amplified c-*erb*B-2 genes may contain point mutations,[65] none has been detected in primary human neoplasms.[51,53,61]

TECHNIQUES FOR DETECTING c-*erb*B-2 ACTIVATION

Detection of c-*erb*B-2 DNA Amplification

Amplification of c-*erb*B-2 DNA is usually detected by DNA dot blot or Southern blot hybridization. In the dot blot method, the extracted DNA is placed directly on a nylon membrane and hybridized with a c-*erb*B-2 DNA probe. In the Southern blot method, the extracted DNA is treated with a restriction enzyme, and the fragments are separated by electrophoresis, transferred to a nylon membrane, and hybridized with a c-*erb*B-2 DNA probe. In both techniques, c-*erb*B-2 amplification is quantified by comparing the intensity (measured by densitometry) of the hybridization bands from the sample with those from control tissue.

Several technical problems may complicate the measurement of c-*erb*B-2 DNA amplification. First, the extracted tumor DNA may be excessively degraded or diluted by DNA from stromal cells.[51] Second, the c-*erb*B-2 DNA probe must be carefully chosen and labeled. For example, oligonucleotide c-*erb*B-2 probes may not be sensitive enough for measuring a low level of c-*erb*B-2 amplification, because diploid copy numbers can be difficult to detect (unpublished data). Third, the total amounts of DNA in the sample and control tissue must be compensated for, often with a probe to an unamplified gene. Many studies have used control probes to genes on chromosome 17, the location of c-*erb*B-2, to correct for possible alterations in chromosome number. Identical results, however, are obtained by using control probes to genes on other chromosomes,[5,63,69] with rare exception.[17] Studies using control probes to the beta-

globin gene must be interpreted with caution, because one allele of this gene is deleted occasionally in breast carcinomas.[3]

Amplification of c-*erb*B-2 DNA was assessed by using the polymerase chain reaction (PCR) in one recent study.[30] Oligoprimers for the c-*erb*B-2 gene and a control gene are added to the sample's DNA, and PCR is performed. If the sample contains more copies of c-*erb*B-2 DNA than of the control gene, the c-*erb*B-2 DNA is replicated preferentially.

Detection of c-*erb*B-2 mRNA Overproduction

Overproduction of c-*erb*B-2 mRNA usually is measured by RNA dot blot or Northern blot hybridization. Both techniques require extraction of RNA but otherwise are analogous to DNA dot blot and Southern blot hybridization. Use of PCR for detection of c-*erb*B-2 mRNA has been described in two recent abstracts.[29,102]

Overproduction of c-*erb*B-2 mRNA can be measured by in situ hybridization. Sections are mounted on glass slides, treated with protease, hybridized with a radiolabeled probe, washed, treated with nuclease to remove unbound probe, and developed for autoradiography. Silver grains are seen only over tumor cells that overproduce c-*erb*B-2 mRNA. Negative control probes are used.[28,29,103] Our experience indicates that these techniques are relatively insensitive for detecting c-*erb*B-2 mRNA overproduction in routinely processed tissue. Although the sensitivity may be increased by modifications that allow simultaneous detection of c-*erb*B-2 DNA and mRNA, in situ hybridization still is cumbersome and expensive (unpublished data).

All of the above c-*erb*B-2 mRNA detection techniques have several problems that make them more difficult to perform than techniques for detecting DNA amplification. One major problem is the rapid degradation of RNA in tissue that is not immediately frozen or fixed. In addition, during the detection procedure, RNA can be degraded by RNase, a ubiquitous enzyme, which must be eliminated meticulously from laboratory solutions. Third, control probes to genes that are uniformly expressed in the tissue of interest need to be carefully selected.

Detection of c-*erb*B-2 Protein Overproduction

The most accurate methods for detecting c-*erb*B-2 protein overproduction are the Western blot method and immunoprecipitation. Both techniques can document the binding specificity of various antibodies against c-*erb*B-2 protein. In Western blot studies, protein is extracted from the tissue, separated by electrophoresis (according to size), transferred to a membrane, and detected by using antibodies to c-*erb*B-2. In immunoprecipitation studies, antibodies against c-*erb*B-2 are added to a tumor lysate, and the resulting protein–antibody precipitate is separated by gel electrophoresis and stained for protein. Both Western blot and immunoprecipitation are useful research tools but currently are not practical for diagnostic pathology. Two recent abstracts have described an enzyme-linked immunosorbent assay (ELISA) for detection of c-*erb*B-2 protein.[29,43]

Overproduction of c-*erb*B-2 protein is most commonly assessed by various immunohistochemical techniques. These procedures often generate conflicting results, which are explained at least partially by three factors. First, various studies have used different polyclonal and monoclonal antibodies. Because some polyclonal antibodies recognize weak bands in addition to the c-*erb*B-2 protein band on Western blot or immunoprecipitation, the results of these studies should be interpreted with caution.[42,43,47,48] Even some monoclonal antibodies immunoprecipitate protein bands in addition to c-*erb*B-2 (p185).[39,50,51] Second, tissue fixation contributes to variability between studies. For example, some antibodies detect c-*erb*B-2 protein only in frozen tissue and do not react in fixed tissue. In general, formalin fixation diminishes the sensitivity of immunohistochemical methods and decreases the number of reactive cells.[51,52] When Bouin's fixative is used, there may be a higher percentage of positive cases.[52] Third, minimal criteria for interpreting immunohistochemical staining are generally lacking. Although there is general agreement that distinct crisp cytoplasmic membrane staining is diagnostic for c-*erb*B-2 activation in breast carcinoma, the number of positive cells and the staining intensity required to diagnose c-*erb*B-2 protein overproduction varies from study to study and from antibody to antibody. Degradation of c-*erb*B-2 protein is not a problem because it can be detected in intact form more than 24 hours after tumor resection without fixation or freezing.[44]

ACTIVATION OF c-*erb*B-2 IN BREAST LESIONS

Incidence of c-*erb*B-2 Activation

Most studies of c-*erb*B-2 oncogene activation do not specify histological subtypes of infiltrating breast carcinoma. Amplification of c-*erb*B-2 DNA was found in 19.1 percent (519 of 2715) of invasive carcinomas in 25 studies (Table 1), and c-*erb*B-2 mRNA or protein overproduction was detected in 20.9 percent (568 of 2714) of invasive carcinomas in 20 studies. Twelve studies have documented c-*erb*B-2 mRNA or protein overproduction in 15 percent (68 of 604) of carcinomas that lacked c-*erb*B-2 DNA amplification.

The incidence of c-*erb*B-2 activation in infiltrating breast carcinoma varies with the histological subtype. Approximately 22 percent (142 of 650) of infiltrating ductal carcinomas have c-*erb*B-2 activation, as expected from the above data. Other variants of breast carcinoma with frequent c-*erb*B-2 activation are inflammatory carcinoma (62 percent, 54 of 87), Paget's disease (82 percent, 9 of 11), and medullary carcinoma (22 percent, 5 of 23). In contrast, c-*erb*B-2 activation is infrequent in infiltrating lobular carcinoma (7 percent, 5 of 73) and tubular carcinoma (7 percent, 1 of 15).

The c-*erb*B-2 protein overproduction is present in 44 percent (44 of 100) of ductal carcinomas in situ and especially comedocarcinoma in situ (68 percent, 49 of 72). The micropapillary type of ductal carcinoma in situ also tends to have c-*erb*B-2 activation,[40,54,63] especially if larger cells are present. The greater fre-

TABLE 1. c-erbB-2 ACTIVATION IN MALIGNANT HUMAN BREAST NEOPLASMS

Histological Diagnosis	c-erbB-2 DNA Amplification[a]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction[b]
Carcinoma, not otherwise specified	146/528,[81] 52/310,[17] 52/291,[100] 28/176,[67] 17/157,[113] 22/141,[88] 14/136,[37] 12/122,[4] 19/103,[79] 15/95,[80] 16/86,[111] 17/73,[77] 16/66,[62] 6/61,[50] 11/57,[52] 10/57,[55] 13/51,[13] 8/49,[81] 10/38,[82] 12/38,[94] 1/25,[15] 7/24,[91] 7/15,[91] 7/10,[96] 2/10[107]	42/180,[89] 49/126,[36] 19/62,[68] 19/57,[60] 3/11,[89] 6/10,[96] 3/9[91]	118/728,[85b] 58/330,[176] 47/313,[96] 17/195,[11] 32/191,[58] 31/185,[101] 34/102,[42] 24/53,[82b] 23/47,[13] 22/45,[8] 11/36,[94] 7/24,[91] 1/10[91]
Carcinoma, type not specified but lacking c-erbB-2 DNA amplification	—	18/138,[81] 14/73,[34] 8/36,[96] 0/8,[90] 1/4,[91] 0/3[96]	16/231,[176] 18/136,[81] 13/35,[13] 14/29,[82b] 1/28,[82] 3/24,[94] 0/17[91]
Infiltrating ductal carcinoma	21/118,[82] 23/107,[94] 17/50,[44] 7/37[96] 14/53 (comedo-carcinoma)[14] 3/33 (tubuloductal carcinoma)[16]	35/85[94]	22/137,[40] 14/83,[93] 8/34[96]

Inflammatory carcinoma	33/80,[35] 3/6[52]	46/75[35]	5/6[52b]
Paget's disease	—	—	5/6,[40] 2/3,[54] 2/2[62]
Tubular carcinoma	0/5,[18] 0/1[53]	—	1/9[40]
Medullary carcinoma	2/4,[18] 0/1[34]	0/1[34]	1/12,[40] 1/3,[58] 1/2,[62] 0/1[30]
Mucinous carcinoma	0/1,[18] 0/1[53]	—	1/2[54]
Invasive papillary carcinoma	0/2[53]	—	—
Infiltrating lobular carcinoma	1/15,[18] 0/6[34]	1/5[34]	2/27,[62] 0/12,[40] 0/9,[30] 1/5[58]
Mammary fibrosarcoma	0/1[53]	—	—
"Benign cystosarcoma"	—	—	0/1[58]
Ductal CIS[c] with minimal invasion	3/5[62]	—	—
Ductal CIS	0/2[34]	1/2[34]	33/74,[40] 10/24[30]
Ductal CIS, solid or comedo type	—	—	20/33,[58] 19/29,[62] 10/10[34]
Ductal CIS, micropapillary type	—	—	10/10[58]
Ductal CIS, micropapillary or cribriform type	—	—	1(local)/14[54]
Ductal CIS, papillary or cribriform type	—	—	0/16,[62] 1/9,[58] 0/3[40]
Lobular CIS	—	—	0/16[40]

[a]Shown as number of cases with activation/number of cases studied; reference is given as a superscript.
[b]These protein studies used Western blots; the rest used immunohistochemical methods.
[c]CIS = carcinoma in situ.

quency of c-*erb*B-2 protein overproduction in comedocarcinoma in situ, compared with infiltrating ductal carcinoma, could be explained by the fact that many infiltrating ductal carcinomas arise from other types of intraductal carcinoma, which show c-*erb*B-2 activation infrequently. Others have speculated that carcinoma in situ with c-*erb*B-2 activation tends to regress or to lose c-*erb*B-2 activation during progression to invasion.[40,58,63] Infiltrating and in situ components of ductal carcinoma, however, usually are similar with respect to c-*erb*B-2 activation,[11,39] although some authors have noted more heterogeneity of the immunohistochemical staining pattern in invasive than in in situ carcinoma.[40,43,58] Activation of c-*erb*B-2 is infrequent in lobular carcinoma in situ. If lesions contain more than one histological pattern of carcinoma in situ, the c-*erb*B-2 protein overproduction tends to occur in the comedocarcinoma in situ but may include other areas of carcinoma in situ.[42,54,68] Overproduction of c-*erb*B-2 protein in ductal carcinoma in situ correlates with larger cell size and a periductal lymphoid infiltrate.[68]

Activation of c-*erb*B-2 has not been identified in benign breast lesions, including fibrocystic disease, fibroadenomas, and radial scars (Table 2). Strong membrane immunohistochemical reactivity for c-*erb*B-2 has not been described in atypical ductal hyperplasia, although weak accentuation of membrane staining has been noted infrequently.[39,42,54] In normal breast tissue, c-*erb*B-2 DNA is diploid, and c-*erb*B-2 is expressed at lower levels than in activated tumors.[34,35,65,68]

These preliminary data suggest that c-*erb*B-2 activation may not be useful for resolving many of the common problems in diagnostic surgical pathology. For example, c-*erb*B-2 activation is infrequent in tubular carcinoma and radial scars. In addition, because c-*erb*B-2 activation is unusual in atypical ductal hyperplasia, cribriform carcinoma in situ, and papillary carcinoma in situ, detection of c-*erb*B-2 activation in these lesions may not be helpful in their differential diagnosis. The histological features of comedocarcinoma in situ, which commonly overproduces c-*erb*B-2, are unlikely to be mistaken for those of benign lesions. Activation of

TABLE 2. c-*erb*B-2 ACTIVATION IN BENIGN HUMAN BREAST LESIONS

Histological Diagnosis	c-*erb*B-2 DNA Amplification*	c-*erb*B-2 mRNA Overproduction	c-*erb*B-2 Protein Overproduction
Fibrocystic disease	0/10[33]	—	0/32,[39] 0/9,[65] 0/8[68]
Atypical ductal hyperplasia	—	—	2(weak)/21,[54] 1(cytoplasmic)/13[39]
Benign ductal hyperplasia	—	—	0/12[39]
Sclerosing adenosis	—	—	0/4[39]
Fibroadenomas	0/16,[34] 0/8,[33] 0/2,[51] 0/1[51]	0/6,[35] 0/3[34]	0/21,[39] 0/10,[65] 0/8,[68] 0/3[42]
Radial scars	—	—	0/22[39]
Blunt duct adenosis	—	—	0/14[39]
"Breast mastosis"	—	0/3[33]	—

*Shown as number of cases with activation/number of cases studied; reference is given as a superscript.

c-*erb*B-2, however, does favor infiltrating ductal carcinoma over infiltrating lobular carcinoma. Further studies of these issues would be useful.

Correlation of c-*erb*B-2 Activation With Pathologic Prognostic Factors

Multiple studies have attempted to correlate c-*erb*B-2 activation with various pathologic prognostic factors (Table 3). Activation of c-*erb*B-2 was correlated with lymph node metastasis in 8 of 23 series, with higher histological grade in 6 of 17 series, and with higher stage in 4 of 14 series. Large tumor size was not associated with c-*erb*B-2 activation in most studies (11 of 14). Tetraploid DNA content and low proliferation, measured by Ki-67, have been suggested as prognostic factors and may correlate with c-*erb*B-2 activation.[67]

Correlation of c-*erb*B-2 Activation With Clinical-Prognostic Factors

Various studies have attempted also to correlate c-*erb*B-2 activation with clinical features that may predict a poor outcome (Table 4). Activation of c-*erb*B-2 correlated with absence of estrogen receptors in 10 of 23 series and with absence of progesterone receptors in 6 of 18 series. In most studies, patient age did not correlate with c-*erb*B-2 activation, and, in the rest of the reports, c-*erb*B-2 activation was associated with either younger or older ages.

Correlation of c-*erb*B-2 Activation With Patient Outcome

Slamon et al[70,81] first showed that amplification of the c-*erb*B-2 oncogene independently predicts decreased survival of patients with breast carcinoma. The correlation of c-*erb*B-2 amplification with poor outcome was nearly as strong as the correlation of number of involved lymph nodes with poor outcome. Slamon et al also reported that c-*erb*B-2 amplification is an important prognostic indicator only in patients with lymph node metastasis.[70,81]

A large number of subsequent studies also attempted to correlate c-*erb*B-2 activation with prognosis (Table 5). In 12 series, there was a correlation between c-*erb*B-2 activation and tumor recurrence or decreased survival. In five of these series, the predictive value of c-*erb*B-2 activation was reported to be independent of other prognostic factors. In contrast, 18 series did not confirm the correlation of c-*erb*B-2 activation with recurrence or survival. Four possible explanations for this controversy are discussed below.

One problem is that c-*erb*B-2 amplification correlates with prognosis mainly in patients with lymph node metastasis. As summarized in Table 5, most studies of patients with axillary lymph node metastasis showed a correlation of c-*erb*B-2 activation with poor outcome. In contrast, most studies of patients without axillary metastasis have not demonstrated a correlation with patient outcome. Table 6 summarizes the studies in which all patients (with and without axillary metastasis) were considered as one group. There is a trend for studies with a higher percentage of metastatic cases to show an association between c-*erb*B-2 activation and poor outcome. Thus, most of the current evidence suggests that c-*erb*B-2 activation has prognostic value only in patients with metastasis to lymph nodes.

TABLE 3. CORRELATION OF c-*erb*B-2 ACTIVATION WITH PATHOLOGIC PROGNOSTIC FACTORS IN BREAST CARCINOMA

Prognostic Factor	P[a]	c-*erb*B-2 DNA Amplification[b]	c-*erb*B-2 mRNA Overproduction	c-*erb*B-2 Protein Overproduction[c]
Metastasis to axillary lymph nodes	<0.05	(118)[35] (105)[34] (49)[21]	(104)[35] (82)[34] (9)[21]	(350)[150] (36)[13]
	0.05–0.15	(103)[79] (88)[79] (58)[111]	—	(189)[92]
	>0.15	(279)[17] (176)[57] (157)[113] (122)[4] (85)[90] (50)[83] (50)[44] (47)[13] (41)[93]	(50)[59]	(329)[170] (261)[90] (195)[11] (185)[101] (102)[39] (50)[52b]
Larger size	<0.05	(280)[17]	—	(330)[170] (189)[92]
	0.05–0.15	(86)[79]	—	—
	>0.15	(176)[57] (157)[113] (103)[79] (84)[77] (58)[111] (45)[31]	(51)[59]	(350)[85a] (185)[101] (34)[22]
Higher stage	<0.05	(300)[17] (64)[77] (58)[111]	—	(349)[17a]
	0.05–0.15	(56)[52]	—	—
	>0.15	(176)[57] (157)[113] (84)[90] (61)[50] (53)[21] (52)[57] (41)[93]	—	(102)[39] (56)[52a]
Higher histological grade	<0.05	(47)[13] (15)[31]	(53)[85]	(178)[101] (168)[11] (38)[15]
	0.05–0.15	—	—	—
	>0.15	(122)[4] (113)[34] (85)[90] (58)[111] (50)[44] (41)[93]	(66)[13] (65)[35]	(290)[58] (189)[92] (102)[39]

[a]A correlation is statistically significant at <0.05, equivocal at best between 0.05 and 0.15, and not statistically significant at >0.15.
[b]Numbers inside parentheses are the number of patients in an individual study; superscript is the reference. Some studies analyzed more than one group of patients.
[c]By Western blot method; all other protein studies used immunohistochemical methods.

TABLE 4. CORRELATION OF c-*erb*B-2 ACTIVATION WITH CLINICAL PROGNOSTIC FACTORS IN BREAST CARCINOMA

Prognostic Factor	p^a	c-*erb*B-2 DNA Amplification[b]	c-*erb*B-2 mRNA Overproduction	c-*erb*B-2 Protein Overproduction[c]
Absence of estrogen receptors	<0.05	(253)[109] (141)[35] (109)[34] (86)[79] (50)[44] (47)[13]	(104)[36]	(350)[35a] (330)[17a] (185)[101]
	0.05–0.15	—	—	—
	>0.15	(157)[113] (122)[4] (103)[79] (95)[80] (64)[77] (61)[50] (58)[111] (53)[21] (51)[52] (41)[20]	(180)[69] (62)[55] (62)[35] (57)[50]	(290)[58] (172)[11] (51)[52c] (38)[13]
Absence of progesterone receptors	<0.05	(253)[109] (141)[35] (109)[34] (50)[44]	—	(350)[35a] (306)[17a]
	0.05–0.15	(86)[79] (49)[62]	—	—
	>0.15	(157)[113] (122)[4] (103)[79] (84)[77]	(180)[69] (103)[36] (62)[55] (56)[38]	(90)[11] (49)[52c]
Age (menopausal status)	<0.05	—	—	(younger: 330)[17a] (older: 58)[52c]
	0.05–0.15	(younger: 86)[79]	—	—
	>0.15	(230)[17] (176)[87] (157)[113] (122)[4] (116)[34] (103)[79] (95)[80] (64)[77] (58)[111] (56)[52] (53)[21] (49)[13] (41)[20] (15)[21]	(62)[55]	(350)[35a] (290)[58] (189)[62] (162)[11] (45)[62]

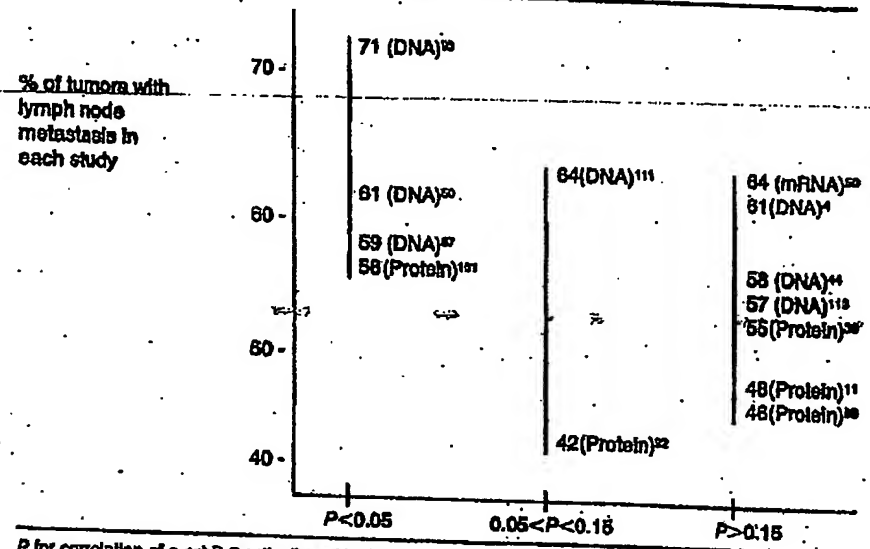
[a]A correlation is statistically significant at <0.05, equivocal at best between 0.05 and 0.15, and not statistically significant at >0.15
[b]Numbers inside parentheses are the number of patients in an individual study; superscript is the reference. Some studies analyzed more than one group of patients.
[c]By Western blot method; all other protein studies used immunohistochemical methods.

TABLE 5. CORRELATION OF c-*erb*B-2 ACTIVATION WITH OUTCOME IN PATIENTS WITH BREAST CARCINOMA

		Number of Patients				
p[a]	Type of c-*erb*B-2 Activation[b]	Total	With Metastasis to Axillary Lymph Nodes	No Metastasis	Statistical Analysis[c]	Reference
<0.05	DNA	176			M	87
<0.05	DNA	61			U	50
<0.05	DNA	57			U	65
<0.05	DNA	41			U	93
<0.05	mRNA	62			U	85
<0.05	Protein	102			M	101
<0.05	DNA		345		M	81
<0.05	DNA		120		U	17
<0.05	DNA		91		U	87
<0.05	DNA		88		M	79
<0.05	Protein-WB		350		M	85
<0.05	Protein		62	44	U	101
0.05–0.15	DNA	57			U	111
0.05–0.15	Protein	189			M	92
0.05–0.15	Protein		120		U	86
>0.15	DNA	130			U	113
>0.15	DNA	122			M	4
>0.15	DNA	50			U	44
>0.15	mRNA	57			U	50
>0.15	Protein	290			M	86
>0.15	Protein	195			U	11
>0.15	Protein	102			U	39
>0.15	Protein		137		U	17
>0.15	DNA			181	M	81
>0.15	DNA			159	U	17
>0.15	DNA			73	U	87
>0.15	Protein-WB			378	U	86
>0.15	Protein-WB			192	U	17
>0.15	Protein			141	U	86
>0.15	Protein			41	U	40

[a]The endpoints of these studies were tumor recurrence or decreased survival or both. Correlation between c-*erb*B-2 activation and a poorer patient outcome is statistically significant at <0.05, is of equivocal significance at 0.05 to 0.15, and is not significant at >0.15.
[b]Shown as variable measured. Letters "WB" indicate assay by Western blot; the other protein studies used immunohistochemical methods.
[c]M = multivariate statistical analysis; U = univariate statistical analysis.

TABLE 6. PERCENTAGE OF BREAST CARCINOMAS WITH METASTASIS COMPARED WITH PROGNOSTIC SIGNIFICANCE OF c-erbB-2 ACTIVATION



P for correlation of c-*erb*B-2 activation with patient outcome.
Each study's percentage of breast carcinomas with metastasis is compared with the correlation between c-*erb*B-2 activation and outcome. These data include only those studies that considered, as one group, all breast cancer patients, whether or not they had axillary metastasis. Superscripts are the references. In parentheses are the types of c-*erb*B-2 activation. *P* values are interpreted as in Table 3.

A second problem is that various types of breast carcinoma are grouped together in many survival studies. Because the current literature suggests that c-*erb*B-2 activation is infrequent in lobular carcinoma, studies that combine infiltrating ductal and lobular carcinomas may dilute the prognostic effect of c-*erb*B-2 activation in ductal tumors. In addition, most studies do not analyze inflammatory breast carcinoma separately. This condition frequently shows c-*erb*B-2 activation and has a worse prognosis than the usual mammary carcinoma, but it is an uncommon lesion.

A third potential problem is the paucity of studies that attempt to correlate c-*erb*B-2 activation with clinical outcome in subsets of breast carcinoma without metastasis. Two recent abstracts reported that in patients without lymph node metastasis who had various risk factors for recurrence (such as large tumor size and absence of estrogen receptors), c-*erb*B-2 overexpression predicted early recurrence.[23,87] In patients with ductal carcinoma in situ, one small study found no association between tumor recurrence and c-*erb*B-2 activation.[40]

A fourth problem is the lack of data regarding whether the prognosis correlates better with c-*erb*B-2 DNA amplification or with mRNA or protein overproduction. Most studies that find a correlation between c-*erb*B-2 activa-

tion and poor patient outcome measure c-*erb*B-2 DNA amplification (Table 5), and breast carcinoma patients with greater amplification of c-*erb*B-2 may have poorer survival.[78,81] Recent studies suggest that amplification has more prognostic power than overproduction,[17,34,53] but the clinical significance of c-*erb*B-2 overproduction without DNA amplification deserves further research.[17,53] Few studies have attempted to correlate patient outcome with c-*erb*B-2 mRNA overproduction, and many studies of c-*erb*B-2 protein overproduction use relatively less reliable methods such as immunohistochemical studies with polyclonal antibodies.

Comparison of c-*erb*B-2 Activation With Other Oncogenes in Breast Carcinoma

Other oncogenes that may have prognostic implications in human breast cancer are reviewed elsewhere.[74,103] This section will be restricted to a comparison between the clinical relevance of c-*erb*B-2 and these other oncogenes.

The c-*myc* gene is often activated in breast carcinomas, but c-*myc* activation generally has less prognostic importance than c-*erb*B-2 activation.[31,34,77,97,99] One study found a correlation between increased mRNAs of c-*erb*B-2 and c-*myc*, although other reports have not confirmed this.[34,103] Subsequent research, however, could demonstrate a subset of breast carcinomas in which c-*myc* has more prognostic importance than c-*erb*B-2.

The gene c-*erb*B-1 for the epidermal growth factor receptor (EGFR) is homologous with c-*erb*B-2 but is infrequently amplified in breast carcinomas.[70] Overproduction of EGFR, however, occurs more frequently than amplification and may correlate with a poor prognosis. In studies that have examined both c-*erb*B-2 and EGFR in the same tumor, c-*erb*B-2 has a stronger correlation with poor prognostic factors.[35,53] Studies have tended to show no correlation between amplification of c-*erb*B-2 and c-*erb*B-1 or overproduction of c-*erb*B-2 and EGFR, although at the molecular level EGFR mediates phosphorylation of c-*erb*B-2 protein.[51,52,61,62,103] Recent reviews describe EGFR in breast carcinoma.[49,103]

The genes c-*erb*A and *ear*-1 are homologous to the thyroid hormone receptor, and they are located adjacent to c-*erb*B-2 on chromosome 17. These genes are frequently coamplified with c-*erb*B-2 in breast carcinomas. The absence of c-*erb*A expression in breast carcinomas, however, is evidence against an important role for this gene in breast neoplasia.[29] Amplification of c-*erb*B-2 can occur without *ear*-1 amplification, and these tumors have a decreased survival that is similar to tumors with both c-*erb*B-2 and *ear*-1 amplification.[97] Consequently, c-*erb*B-2 amplification seems to be more important than amplification of c-*erb*A or *ear*-1.

Other genes also have been compared with c-*erb*B-2 activation in breast carcinomas. One study found a significant correlation between increased c-*erb*B-2 mRNA and increased mRNAs of *fos*, platelet-derived growth factor chain A, and Ki-*ras*.[103] Allelic deletion of c-Ha-*ras* may indicate a poorer prognosis in breast carcinoma,[31] but it has not been compared with c-*erb*B-2 activation. Some studies have suggested a correlation between advanced stage or recurrence of breast carcinoma and activation of any one of several oncogenes.[31,103]

ACTIVATION OF c-erbB-2 IN NON-MAMMARY TISSUES

Incidence of c-erbB-2 Activation in Non-Mammary Tissues

Table 7 summarizes the normal tissues in which c-erbB-2 expression has been detected, usually with immunohistochemical methods using polyclonal anti-

TABLE 7. PRESENCE OR ABSENCE OF c-erbB-2 mRNA OR c-erbB-2 PROTEIN IN NORMAL HUMAN TISSUES

Tissues With c-erbB-2 mRNA	Tissues Producing c-erbB-2 Protein[a]	Tissues Lacking c-erbB-2 mRNA	Tissues Lacking c-erbB-2 Protein
Skin[24]	Epidermis[36]		
	External root sheath[36]		
	Eccrine sweat gland[36]		
	Fetal oral mucosa[52]		Postnatal oral mucosa[52]
	Fetal esophagus[52]		Postnatal esophagus[52]
Stomach[24]	Stomach[22,52]		
	Fetal intestine[22a]		
Jejunum[24]	Small intestine[22,52]		
Colon[24]	Colon[22,52]		
Kidney[24]	Fetal kidney[52a]	Kidneys[104]	Glomerulus[52]
			Postnatal Bowman's capsule[52]
	Fetal proximal tubule[52]		Postnatal proximal tubule[52]
	Distal tubule[52]		
	Fetal collecting duct[52]		Postnatal collecting duct[52]
	Fetal renal pelvis[52]		Postnatal renal pelvis[52]
	Fetal ureter[52]		Postnatal fetal ureter[52]
Liver[24]	Hepatocytes[22]		Liver[22,25]
	Pancreatic acini[22]		
	Pancreatic ducts[22,52]		
	Endocrine cells of islets of Langerhans[22]		Pancreatic islets[52]
Lung[24]	Fetal trachea[52]		Postnatal trachea[52]
	Fetal bronchioles[52]		Postnatal bronchioles[52]
	Bronchioles[36]		
			Postnatal alveoli[52,53]
Fetal brain[24]			Postnatal brain[52]
	Fetal ganglion cells[52]		Postnatal ganglion cells[52]
Thyroid[1]			
Uterus[24]			
	Ovary[12]		
	Blood vessels[42]		Endothelium[52]
Placenta[24]			
			Adrenocortical cells[52]
			Postnatal thymus[52]
			Fibroblasts[52]
			Smooth muscle cells[52]
			Cardiac muscle cells[52]

[a]This protein study used Western blots; the rest used immunohistochemical methods.

bodies. Only a few studies have been performed, and some of these do not demonstrate convincing cell membrane reactivity in the published photographs. The interpretations in these studies, however, are listed, with the caveat that these findings should be confirmed by immunoprecipitation or Western or RNA blots. Production of c-*erb*B-2 has been identified in normal epithelium of the gastrointestinal tract and skin. Discrepancies regarding c-*erb*B-2 protein in other tissues could be due, at least in part, to differences in techniques.

The data on c-*erb*B-2 activation in various non-mammary neoplasms should be interpreted with caution, because only small numbers of tumors have been studied, usually by immunohistochemical methods using polyclonal antibodies. Studies using cell lines have been excluded, because cell culture can induce amplification and overexpression of other genes, although this has not been documented for c-*erb*B-2.

Activation of c-*erb*B-2 has been identified in 32 percent (64 of 203) of ovarian carcinomas in eight studies (Table 8). One abstract[85] stated that ovarian carcinomas contained significantly more c-*erb*B-2 protein than ovarian non-epithelial malignancies. Another report[51] showed that 12 percent of ovarian carcinomas had c-*erb*B-2 overproduction without amplification.

Activation of c-*erb*B-2 has been identified in 20 percent (40 of 198) of gastric adenocarcinomas in seven studies, including 33 percent (21 of 64) of

TABLE 8. c-*erb*B-2 ACTIVATION IN HUMAN GYNECOLOGIC TUMORS[a]

Tumor Type	c-*erb*B-2 DNA Amplification	c-*erb*B-2 mRNA Overproduction	c-*erb*B-2 Protein Overproduction
Ovary—carcinoma, not otherwise specified	31/120,[51] 1/11,[57] 0/5,[107] 0/5,[84] 0/3,[112] 0/2,[72] 0/1[110]	23/67[51]	23/73,[12] 36/72[51]
Ovary—serous (papillary) carcinoma	2/7,[110] 1/7,[112] 0/6[72]	—	—
Ovary—endometrioid carcinoma	0/3[110]	—	—
Ovary—mucinous carcinoma	1/2,[110] 0/1[72]	—	—
Ovary—clear cell carcinoma	0/2,[112] 0/1[72]	—	—
Ovary—mixed epithelial carcinoma	0/2[72]	—	—
Ovary—endometrioid borderline tumor	0/1[72]	—	—
Ovary—mucinous borderline tumor	0/3[72]	—	—
Ovary—serous cystadenoma	0/4[72]	—	—
Ovary—mucinous cystadenoma	0/2[72]	—	—
Ovary—sclerosing stromal tumor	0/1[72]	—	—
Ovary—fibrothecoma	0/1[72]	—	—
Uterus—endometrial adenocarcinoma	0/4,[84] 0/1[110]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

intestinal or tubular subtypes and 9 percent (4 of 47) of diffuse or signet ring cell subtypes (Table 9). Activation of c-*erb*B-2 has been detected in 2 percent (6 of 281) of colorectal carcinomas, although an additional immunohistochemical study detected c-*erb*B-2 protein in seven of eight tissues fixed in Bouin's solution. One study found greater immunohistochemical reactivity for c-*erb*B-2 protein in colonic adenomatous polyps than in the adjacent normal epithelium, using Bouin's fixative. Lesions with anaplastic features and progression to invasive carcinoma tended to show decreased immunohistochemical reactivity for c-*erb*B-2 protein.[92] Hepatocellular carcinomas (12 of 14 cases) and cholangiocarcinomas (46 of 63 cases) reacted with antibodies against c-*erb*B-2 in one study, but some of these "positive" cases showed only diffuse cytoplasmic staining, which

TABLE 9. c-erbB-2 ACTIVATION IN HUMAN GASTROINTESTINAL TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 Protein Overproduction
Esophagus—squamous cell carcinoma	0/1[107]	0/1[91]
Stomach—carcinoma, poorly differentiated	0/22[108]	—
Stomach—adenocarcinoma	2/24,[94] 2/9,[107] 2/8,[111] 2/8,[57] 0/1[108]	4/27,[29] 3/10[91]
Stomach—carcinoma, intestinal or tubular type	5/10[108]	16/54[29]
Stomach—carcinoma, diffuse or signet ring cell type	0/2[108]	4/45[29]
Colorectum—carcinoma	2/49,[94] 1/45,[111] 1/45,[57] 1/45,[60] 0/40,[91] 0/32,[107] 0/3[92]	1/22,[58] 7/8[22b]
Colon—villous adenoma	0/1[60]	—
Colon—tubulovillous adenoma	0/5[60]	—
Colon—tubular adenoma	0/7[60]	19/19[22b]
Colon—hyperplastic polyp	0/1[60]	—
Intestine—leiomyosarcoma	—	0/1[91]
Hepatocellular carcinoma	0/12[111]	12/14,[58] 0/2[91]
Hepatoblastoma	0/1[57]	—
Cholangiocarcinoma	—	46/63[95]
Pancreas—adenocarcinoma	—	2/80,[21c] 0/2[91]
Pancreas—acinar carcinoma	—	0/1[41]
Pancreas—clear cell carcinoma	—	0/2[41]
Pancreas—large cell carcinoma	—	0/3[41]
Pancreas—signet ring carcinoma	—	0/1[41]
Pancreas—chronic inflammation	—	0/14[41c]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods. No studies analyzed for c-*erb*B-2 mRNA.
[b]Tissues fixed in Bouin's solution.
[c]Only cases with distinct membrane staining are interpreted as showing c-*erb*B-2 overproduction.

TABLE 10. c-*erb*B-2 ACTIVATION IN HUMAN PULMONARY TUMORS[a]

Tumor Type	c-*erb*B-2 DNA Amplification	c-*erb*B-2 Protein Overproduction
Non-small cell carcinoma	2/60,[75] 0/60[81]	1/84[58]
Epidermoid carcinoma	0/13,[82] 0/10,[57] 0/5[57]	3/5[58]
Adenocarcinoma	0/21,[58] 1/13,[20] 0/7,[111] 0/7,[57] 0/3[107]	4/12[58]
Large cell carcinoma	0/9,[82] 0/6[58]	—
Small cell carcinoma	—	0/26,[58] 0/3[58]
Carcinoid tumor	0/1[82]	0/3[58]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods. No studies analyzed for c-*erb*B-2 mRNA.

does not indicate c-*erb*B-2 activation in breast neoplasms.[85] Also, some pancreatic carcinomas and chronic pancreatitis tissue had cytoplasmic immunohistochemical reactivity for c-*erb*B-2 protein, in addition to the rare case of pancreatic adenocarcinoma with distinct cell membrane staining.[42]

Tables 10 through 14 summarize the studies of c-*erb*B-2 activation in other neoplasms. The c-*erb*B-2 oncogene is not activated in most of these tumors. Activation of c-*erb*B-2 has been detected in 1 percent (4 of 299) of pulmonary non-small cell carcinomas in nine studies, although one additional report[58] found c-*erb*B-2 protein overproduction in 41 percent (7 of 17). Renal cell carcinoma had c-*erb*B-2 activation in 7 percent (2 of 30) in four studies. Overproduction of c-*erb*B-2 protein was described in one transitional cell carcinoma of the urinary bladder, a grade 2 papillary lesion.[58] Squamous cell carcinoma and basal cell carcinoma of the skin may contain c-*erb*B-2 protein, but it is not clear

TABLE 11. c-*erb*B-2 ACTIVATION IN HUMAN HEMATOLOGIC PROLIFERATIONS[a]

Tumor Type	c-*erb*B-2 DNA Amplification	c-*erb*B-2 mRNA Overproduction	c-*erb*B-2 Protein Overproduction
Hematologic malignancies	0/23[119]	—	—
Malignant lymphoma	0/9,[57] 0/3[107]	0/1[1]	0/15[81]
Acute leukemia	0/14[57]	—	—
Acute lymphoblastic leukemia	0/1[107]	—	—
Acute myeloblastic leukemia	0/3[107]	—	—
Chronic leukemia	0/19[57]	—	—
Chronic lymphocytic leukemia	0/6[107]	—	—
Chronic myelogenous leukemia	0/8[107]	—	—
Myeloproliferative disorder	0/1[57]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

TABLE 12. c-*erb*B-2 ACTIVATION IN HUMAN TUMORS OF SOFT TISSUE AND BONE[a]

Tumor Type	c-*erb*B-2 DNA Amplification
Sarcoma	0/10,[111] 0/8[57]
Malignant fibrous histiocytoma	0/1[107]
Liposarcoma	0/3[107]
Pleomorphic sarcoma	0/1[107]
Rhabdomyosarcoma	0/1[107]
Osteogenic sarcoma	0/2,[107] 0/2[57]
Chondrosarcoma	0/1[107]
Ewing's sarcoma	0/1[57]
Schwannoma	0/1[57]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. No studies analyzed for c-*erb*B-2 mRNA or c-*erb*B-2 protein.

whether the protein level is increased over that of normal skin.[56] Thyroid carcinomas and adenomas can have low levels of increased c-*erb*B-2 mRNA. One abstract described low-level c-*erb*B-2 DNA amplification in one of ten salivary gland pleomorphic adenomas.[48]

Correlation of c-*erb*B-2 Activation With Patient Outcome

Very few studies have attempted to correlate c-*erb*B-2 activation in non-mammary tumors with outcome. Slamon et al[31] showed that c-*erb*B-2 amplification or overexpression in ovarian carcinomas correlates with decreased survival, especially when marked activation is present. However, they did not report the stage, histological grade, or histological subtype of these neoplasms. Another study of stages III and IV ovarian carcinomas found a correlation between decreased survival and c-*erb*B-2 protein overproduction, but not between survival and histological grade.[13] One abstract stated that c-*erb*B-2 protein overproduction in 10 of 16 pulmonary adenocarcinomas correlated with decreased disease-free interval.[70] Another abstract described a tendency for immunohisto-

TABLE 13. c-*erb*B-2 ACTIVATION IN HUMAN TUMORS OF THE URINARY TRACT[a]

Tumor Type	c-*erb*B-2 DNA Amplification	c-*erb*B-2 mRNA Overproduction	c-*erb*B-2 Protein Overproduction
Kidney—renal cell carcinoma	1/6,[57] 1/4,[107] 0/5[34]	0/16[104]	—
Wilms' tumor	0/4[57]	—	—
Prostate—adenocarcinoma	—	—	0/29[58]
Urinary bladder—carcinoma	—	—	1/48[58]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

TABLE 14. c-*erb*B-2 ACTIVATION IN MISCELLANEOUS HUMAN TUMORS[a]

Tumor Type	c-*erb*B-2 DNA Amplification	c-*erb*B-2 mRNA Overproduction	c-*erb*B-2 Protein Overproduction
Skin—malignant melanoma	—	—	0/10[56]
Skin, head and neck—squamous cell carcinoma	0/7[107]	—	—
Site not stated—squamous cell carcinoma	0/8,[57] 0/2[76]	—	—
Salivary gland—adenocarcinoma	1/1[76]	—	—
Parotid gland—adenoid cystic carcinoma	—	—	0/1[56]
Thyroid—anaplastic carcinoma	0/1[9]	0/1[7]	—
Thyroid—papillary carcinoma	0/5[3]	3(low levels)/5[3]	—
Thyroid—adenocarcinoma	0/1[34]	—	—
Thyroid—adenoma	0/2[3]	1(low levels)/2[3]	—
Neuroblastoma	0/35,[31] 0/9,[57] 0/1[76]	—	—
Meningioma	0/2[57]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

chemical reactivity for c-*erb*B-2 protein to correlate with higher grades of prostatic adenocarcinoma.[97] Additional prognostic studies of ovarian carcinomas and other neoplasms are needed.

SUMMARY

Activation of the c-*erb*B-2 oncogene can occur by amplification of c-*erb*B-2 DNA and by overproduction of c-*erb*B-2 mRNA and c-*erb*B-2 protein. Approximately 20 percent of breast carcinomas show evidence of c-*erb*B-2 activation, which correlates with a poor prognosis primarily in patients with metastasis to axillary lymph nodes. Studies that have attempted to correlate c-*erb*B-2 activation with other prognostic factors in breast carcinoma have reported conflicting conclusions. The pathologic and clinical significance of c-*erb*B-2 activation in other neoplasms is unclear and should be assessed by additional studies.

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